



Antiviral therapies on the horizon for influenza

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Adequate response to severe influenza infections or pandemic outbreaks requires two complementary strategies: preventive vaccination and antiviral therapy. The existing influenza drugs, M2 blockers and neuraminidase inhibitors, show modest clinical efficacy and established or potential resistance. In the past three years, several new agents have entered the clinical pipeline and already yielded some promising data from Phase 2 trials. For two main categories, that is, the broadly neutralizing anti-hemagglutinin antibodies and small-molecule inhibitors of the viral polymerase complex, crystallography was instrumental to guide drug development. These structural insights also aid to expand the activity spectrum towards influenza A plus B viruses, or conceive nucleoprotein or polymerase assembly inhibitors. The practice of influenza therapy should radically change in the next decade.

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Introduction

The annual influenza epidemics cause significant morbidity and mortality, particularly in elderly or frail individuals. Worldwide, the number of severe influenza infections is about 5 million each year leading to 500 000 deaths. About 75% of these fatalities are caused by influenza A viruses; the other 25% is due to influenza B virus. The widely recommended influenza vaccination requires annual updating and is only partially effective in some target populations. Besides, the influenza pandemic of 2009 demonstrated our vulnerability to new and suddenly appearing influenza viruses for which pre-existing immunity is lacking. Globally, the 2009 pandemic influenza virus killed ~300 000 people within only 18 months; its death toll peaked in younger persons [1]. Influenza A viruses circulating in swine or birds can enter the human

population and, hence, are a constant threat. For some circulating avian influenza A viruses, the mortality in humans is notoriously high, that is, ~60% for H5N1 and ~30% for H7N9.

As for antiviral therapy, virus resistance is widespread for the M2 channel blockers amantadine and rimantadine, and can also develop with the neuraminidase inhibitors (NAIs) oseltamivir and, to a lesser degree, zanamivir [2]. These two NAIs were stockpiled by many countries in the context of pandemic preparedness. Two newer analogues, peramivir and laninamivir octanoate, were recently approved in some regions. In 2014, the NAIs became matter of heavy debate after a Cochrane meta-analysis of clinical reports [3] concluded that oseltamivir and zanamivir exert only a small effect in shortening influenza disease in adults. On the other hand, a similarly large meta-analysis of individual patient data [4] determined that oseltamivir does reduce the duration of influenza illness as well as the risk for complications or hospitalization. Likewise, a study involving >29 000 patients who were hospitalized during the 2009 pandemic, demonstrated that the mortality risk was significantly reduced upon early NAI treatment [5]. Hence, NAIs are advocated for patients who are at risk for complicated influenza infections [6•].

We here review the recent progress in antiviral drug development for influenza. The (pre)clinical pipeline contains diverse agents (Table 1) many of which target the viral polymerase complex. Other approaches not covered here are: inhibitors of the viral NS1 protein [7] or host factors involved in virus replication [8], such as the receptor destroying sialidase DAS-181 that is in Phase 2 clinical trials [9].

Next-generation neuraminidase inhibitors and M2 channel blockers

Whereas the four NAIs mentioned above are reversible transition state mimetics of the NA reaction, a new class of 2,3-difluoro sialic acid derivatives was designed to bind to NA in a covalent manner. These analogues displayed favorable activity in influenza virus-infected cell culture and mouse models [10]. The lead compound proved active against the oseltamivir-resistant H275Y mutant form of N1 neuraminidase, and was less affected than zanamivir by the E119G neuraminidase mutation.

To address the issue of M2 blocker resistance, several groups [11] are applying crystallographic and NMR structural insights as well as molecular dynamics simulations to rationally develop new M2 inhibitors with activity against wild-type M2, or the clinically relevant S31N or V27A

Table 1

Prototypic influenza inhibitors in various stages of (pre)clinical development

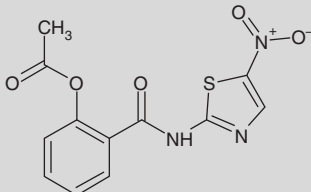
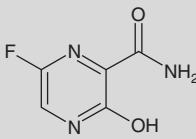
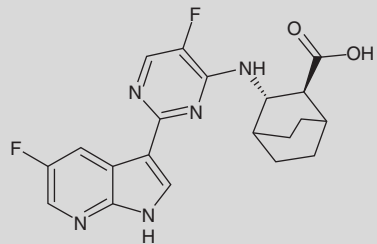
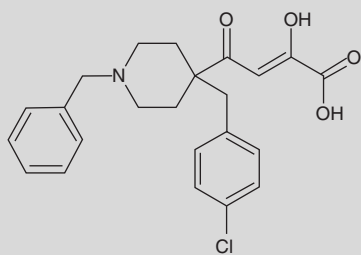
Name and structure	Proposed action principle ^a	Status ^b	References
Hemagglutinin inhibitors — broadly-neutralizing antibodies			
CR6261	Binds to stem region of A/group 1 HAs ^{#§}	Phase 2	[13,14 [*]]
CR8020	Binds to membrane-proximal stem region of A/group 2 HAs ^{#§}	Phase 2	[13,14 [*]]
MHAA4549A	Binds to HA stem epitope conserved in influenza A	Phase 2a	[18,19]
VIS410	Engineered antibody; binds to HA stem epitope conserved in influenza A	Phase 2a	[64]
Hemagglutinin inhibitors — small molecules			
Nitazoxanide	Interferes with virus maturation	Phase 3	[23,24]
			
Polymerase inhibitors			
Favipiravir (T-705)	Nucleobase inhibitor [#] ; causes RNA chain termination and virus mutagenesis	Approved (Japan) Phase 3 (US/EU)	[31–37]
			
VX-787 (JNJ-872)	Blocks the PB2-CBD of influenza A ^{#§}	Phase 2b	[38 ^{**} ,40]
			
L-742,001	Metal-chelating inhibitor of PA endonuclease ^{#§}	Experimental	[48–51]
			

Table 1 (Continued)

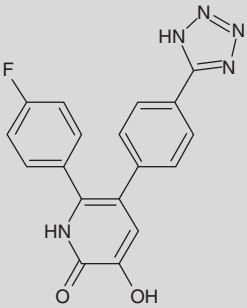
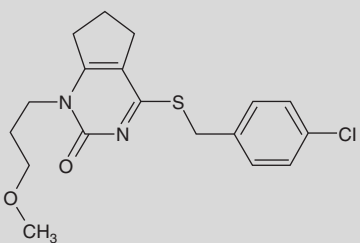
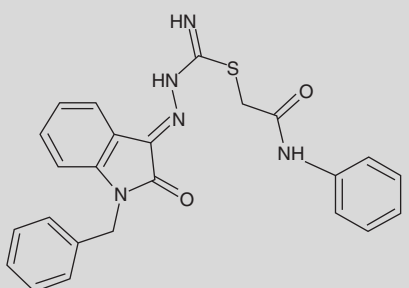
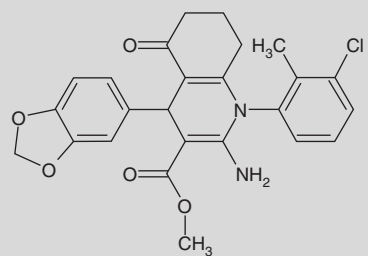
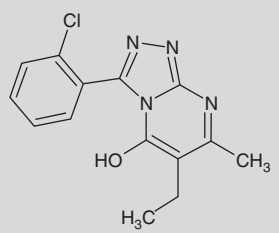
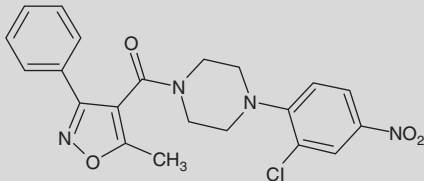
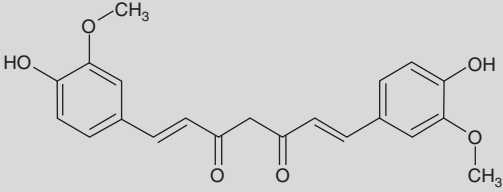
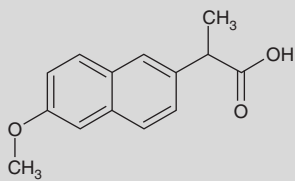
Name and structure	Proposed action principle ^a	Status ^b	References
Compound '7'	Metal-chelating inhibitor of PA endonuclease [§]	Experimental	[52]
			
AL-794 (structure undisclosed)	PA inhibitor	Phase 1	
S-033188 (structure undisclosed)	PA inhibitor	Phase 2	
'367'	Unknown (PB1?) [#]	Experimental	[53]
			
ASN2	Unknown (PB1?) [#]	Experimental	[54]
			
'Compound 1'	Inhibits PA _C -PB1 _N assembly	Experimental	[57,58*]
			
ANA-1	Inhibits PA _C -PB1 _N assembly	Experimental	[59]
			

Table 1 (Continued)

Name and structure	Proposed action principle ^a	Status ^b	References
Nucleoprotein inhibitors			
Nucleozin 	Causes NP aggregates; specific for influenza A ^{#§}	Experimental	[61]
Curcumin 	Interferes with RNA-binding by NP	Experimental	[62*]
Naproxen 	Interferes with RNA-binding by NP	Experimental	[63]

^a The # symbol denotes that the proposed action principle is supported by published resistance studies; the § symbol indicates that cocrystallization data are available.

^b In August 2016.

mutant forms. One approach consists of adding an aromatic ring ('warhead') to the adamantane scaffold [12].

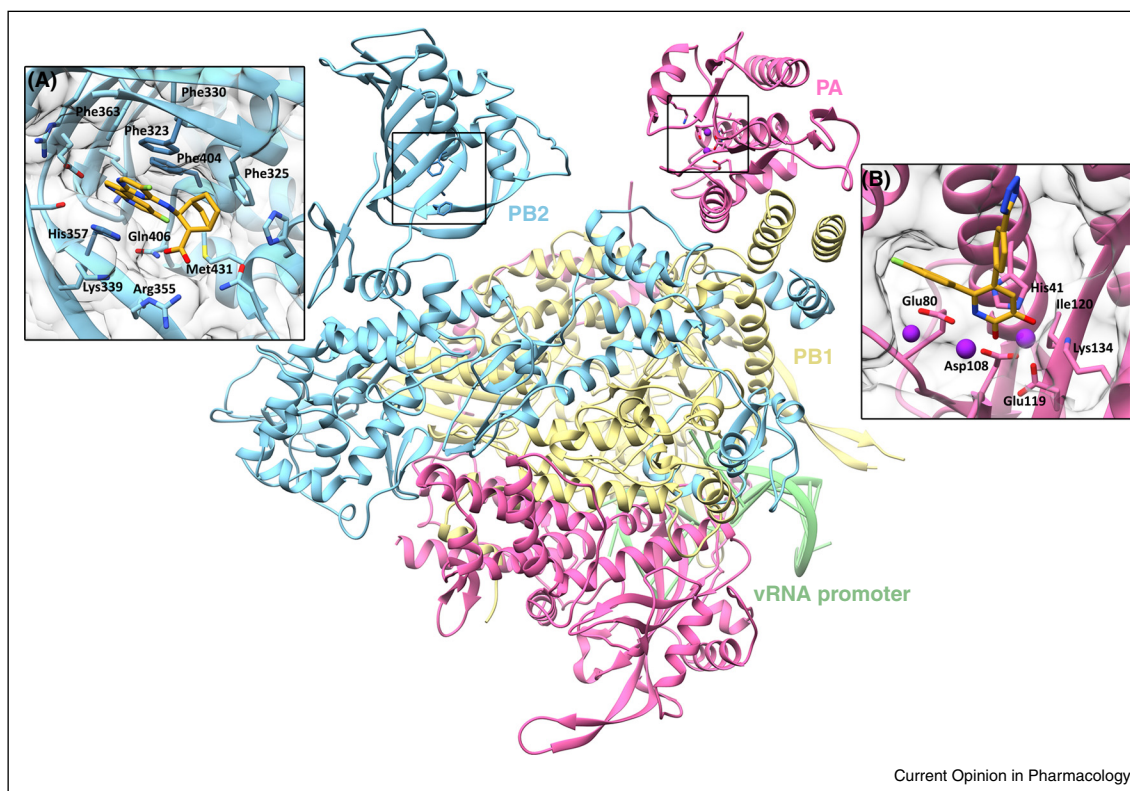
Targeting conserved sites in the viral hemagglutinin

The 18 hemagglutinin (HA) subtypes of influenza A are classified into two phylogenetic groups; the H1, H2 and H5 HAs belong to group 1 whereas the H3 and H7 HAs are in group 2. Also for influenza B, the circulating strains are classified into two lineages (B/Victoria and B/Yamagata). Despite high sequence diversity, some HA regions are conserved within the same group or across both groups of influenza A or B viruses. During recent years, several broadly neutralizing antibodies (bnAbs) (reviewed in [13,14*]) with activity against group 1, group 2, or both influenza A HA groups, were isolated from plasma samples of influenza-infected or influenza-vaccinated individuals. This created the prospect for monoclonal antibody-based therapy. One bnAb (CR9114) recognizes an HA epitope conserved in influenza A and B viruses [15]. The known bnAb binding sites, revealed by cocrystallization, are located at, firstly, an HA globular head region, in most cases the receptor-binding site; secondly, an HA stem domain with a key role in membrane fusion; or thirdly, a membrane-proximal region at the base of the HA stem

[13,14*]. Many bnAbs conferred protection in influenza-infected mouse models, presumably by engaging immune cells via Fc-FcγR interactions [16]. *In vitro*, the propensity to select escape mutants is much higher for anti-globular head than anti-stem bnAbs [13]. This does not exclude, however, that some bnAb binding stem epitopes may be prone to antigenic drift [17]. Several influenza bnAbs (CR8020, CR6261, MEDI8852, CT-P27, MHAB5553A, MHAA4549A and VIS410) are currently undergoing clinical evaluation; for the latter two some limited clinical data were recently disclosed [18,19].

A different agent interfering with the HA-mediated entry process is Flufirvitide-3 (FF-3), a 16-mer HA peptide sequence that entered Phase 1 trials [20]. The design of small-molecule inhibitors towards conserved regions in HA (such as the bnAb binding sites), seems particularly difficult. Various fusion inhibitors were reported to prevent HA refolding at low endosomal pH [21], but their strict subtype-specificity and low resistance barrier have been serious drawbacks for preclinical development. For one H3-specific fusion inhibitor, the binding pocket in the HA stem [22] lies adjacent to the binding site for bnAb CR6261, emphasizing the key role of this region in the processes of HA refolding and fusion.

Figure 1



Structure of the influenza A polymerase heterotrimer when bound to the vRNA promoter. In this open conformation, the enzyme is in its cap-snatching mode with two functional domains, that is, PB2-CBD and PA_N endonuclease, facing each other [pdb: 4WSB] [26^{••}]. (a) Binding pose of the PB2-CBD blocker VX-787 [pdb: 4P1U] [38^{••}]; the marked residues have a critical role in binding VX-787 or the cap analogue 7-methyl-GTP [39]. (b) Binding mode of a PA inhibitor with hydroxypyridinone structure and potent enzyme inhibitory activity [pdb: 4M5U] [52]. The marked residues have a crucial function in binding the divalent metal ions (purple spheres) or catalyzing RNA cleavage [44,45].

An entirely unrelated mechanism applies to nitazoxanide, a thiazolidine compound that was proven effective against influenza in a Phase 2b/3 clinical trial [23]. This small molecule exhibits broad-spectrum activity against influenza A and B besides many other viruses [24]. Its effect on HA appears located at the posttranslational stage, since it was shown to prevent HA transport to the host cell membrane and exit of mature virus particles.

Inhibitors of the viral polymerase complex

Influenza A and B viruses have a single-stranded negative-sense RNA genome divided into eight segments, in which the viral RNA is covered with multiple nucleoprotein molecules and attached to one copy of the viral polymerase. This protein complex contains three subunits, PA, PB1 and PB2, and basically has three functions: firstly, binding of 5'-capped host cell RNAs by the PB2 subunit; secondly, cleavage of these RNAs by the PA endonuclease to produce the primers for viral mRNA synthesis; and thirdly, RNA elongation by the PB1 polymerase [25]. Recently, pioneering crystallographic analyses of the

polymerase heterotrimer of influenza A, B or C [26^{••},27^{••},28^{••},29[•]], have revealed intense inter-subunit interactions and notable structural flexibility of this protein complex, creating the opportunity to design allosteric inhibitors. The PA-PB1-PB2 heterotrimer adopts an open conformation when bound to the vRNA promoter (Figure 1) [26^{••},27^{••}], whereas a closed conformation is seen in the apo-structure [28^{••}] and the protein complex bound to a 5' cRNA fragment [29[•]]. This pronounced difference is related to 'en bloc' rotation of a large part of the PB2 protein, and most probably signifies different functional states of the polymerase complex, with the open conformations representing the 'cap-snatching' and transcription pre-initiation states [25].

Until 2014, structure-aided drug design of influenza polymerase inhibitors was based on crystal structures of partial fragments that became available after 2008. Below, the most successful strategies are briefly explained; a more extensive description can be found elsewhere [30]. We first describe the nucleobase analogue favipiravir, which

was not developed by *in silico* methods and is, thus far, the most advanced polymerase inhibitor for influenza therapy.

Favipiravir (T-705)

This broad RNA virus inhibitor [31] was first reported in 2002. It was approved for influenza therapy in Japan in 2014, and is in Phase 3 trials in the USA and Europe. In one Phase 2 study [19], favipiravir significantly reduced the time to resolution of symptoms without evidence of drug resistance in more than 700 samples tested. Mechanistically, favipiravir acts as a competitive substrate inhibitor of the viral polymerase after its conversion to favipiravir-ribosyl-5'-triphosphate (T-705-RTP) [32]. The low efficiency of its activation [33] appears one reason why favipiravir possesses relatively low antiviral potency. In enzymatic influenza polymerase assays, T-705-RTP mimics both GTP and ATP [34], indicating that it acts as an ambiguous purine nucleotide due to its rotating carboxamide. Although the unmodified ribose allows RNA chain elongation, two consecutive T-705-RTP incorporation events result in 'leaky' chain termination [35]. In cell culture, favipiravir appears to inhibit viral RNA synthesis at high concentrations and produce viral mutagenesis at lower concentrations [36]. Besides having broad activity against influenza A and B, this drug has the advantage of an exceptionally high barrier for drug resistance. Until now, only one mutation (V43I in PB1; obtained in virus-infected cell cultures under ribavirin selection) was found to confer marginal (two-fold) resistance to favipiravir [37]; the relevance of this substitution still needs to be verified in enzymatic polymerase assays.

Inhibitors of cap-binding by PB2

The azaindole compound VX-787 (new code: JNJ-872) is undergoing Phase 2b clinical evaluation after passing successful evaluation in an influenza challenge study in humans [19]. This promising agent displays nanomolar activity in influenza A virus-infected cell cultures and acts by interfering with cap-binding to PB2 [38**].

The crystal structure of the cap-binding domain (CBD) of PB2 in complex with 7-methyl-GTP was first revealed in 2008 [39]. Its recognition mechanism for capped RNA, in which the methylated guanine interacts with two aromatic residues in a cation- π sandwich, resembles that of other cap-binding proteins such as the eukaryotic initiation factor eIF4E. However, since the PB2-CBD has a distinct protein fold with a unique cluster of Phe residues (Figure 1, inset A), highly selective inhibitors are attainable. This is exemplified by VX-787, the product of structure-aided drug design in which an azaindole scaffold initially explored for inhibition of cellular kinases, was rationally optimized towards the influenza PB2-CBD. Its binding mode was clarified by cocrystallization (Figure 1, inset A) and confirmed by resistance selection in cell culture [40]. Development of PB2-CBD inhibitors with equal activity against influenza A and B seems

challenging. Some residues (such as Phe-323) with a crucial role in binding VX-787 in the influenza A PB2-CBD, are not conserved in influenza B [41*]. Also, the PB2-CBD of influenza B is more flexible, explaining its ability to recognize both methylated and unmethylated cap structures, as demonstrated by cocrystallization [41*,42] and biochemical experiments [43].

Inhibitors of the PA endonuclease

In early 2016, two PA inhibitors entered Phase 1 (AL-794) or Phase 2 (S-033188) clinical trials. The N-terminal part of PA (PA_N) carries the catalytic domain for cleavage of the capped host RNAs. Its core region contains one or two divalent metal ions (Mn²⁺ or Mg²⁺) and several residues that are conserved among influenza A and B viruses, given their role in catalysis or metal ion coordination (Figure 1, inset B) [44,45]. This catalytic center is surrounded by distinct hydrophobic pockets that are well suited for drug design [46,47]. Hence, PA inhibitor design is currently built on chemical scaffolds bearing coplanar oxygens with the right geometry to coordinate the divalent metal ion(s), and to which hydrophobic elements have been added. The β -diketo acid derivative L-742,001 [48] serves as a prototype since it is the only reported inhibitor for which target and PA_N binding mode have been confirmed in cell culture-based virus resistance studies [49–51]. Screening efforts with isolated PA_N enzyme have yielded diverse PA inhibitors (see [46,47] for examples). Quite a few of these hit compounds display only weak activity in virus-infected cell cultures, which is likely related to low cell penetration and/or insufficient antiviral selectivity. Crystallographic fragment screening plus hit optimization [52] delivered a hydroxypyridinone-based lead compound with remarkable potency (i.e. IC₅₀ value of 11 nM in a PA_N enzyme assay), suggesting that its metal chelating motif and binding pose within PA_N (Figure 1, inset B) are particularly relevant.

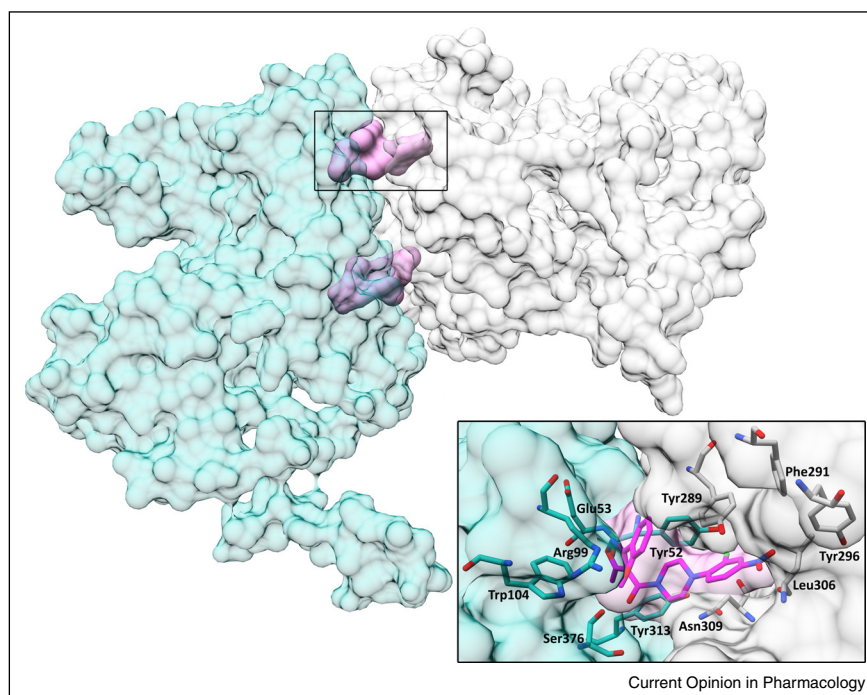
Inhibitors of the PB1 polymerase

The recent revelation of the active site structure of PB1 within the PA-PB1-PB2 heterotrimer [26**,27**] enables to rationally design inhibitors against this RNA-dependent RNA polymerase. Two possible lead compounds, identified by serendipitous screening, are compounds '367' [53] and 'ASN2' [54] which both select for a resistance mutation located at the outside of PB1, i.e. H456P for '367' and S499T for 'ASN2'. The conception of PB1 inhibitors towards the catalytic center for RNA elongation would be facilitated by a crystal structure of PB1 (or the polymerase heterotrimer) in complex with a natural NTP or structural analogue such as T-705-RTP.

Protein-protein interaction (PPI) inhibitors

In the crystal structure of PA_C (the C-terminal domain of PA) in complex with the PB1 N-terminus (PB1_N; residues 1–25), the binding pocket in PA_C resembles a 'dragon's head' that clamps the PB1_N peptide into its 'jaws' [55,56].

Figure 2



Binding mode of nucleozin, a compound that forms bridges between NP monomers resulting in NP aggregation [65]. The inset shows the structure of nucleozin (in pink) and residues aligning its binding pocket [pdb: 3RO5] [61].

The small size and hydrophobic nature of this pocket provided the foundation to design small-molecule PA_C - $PB1_N$ assembly inhibitors, the first of which were reported in 2012 [57]. These prevent the PA_C - $PB1_N$ interaction in biochemical assays and inhibit cell culture growth of influenza A (and, in some cases, also influenza B) virus (see [58[•]] and references therein). Compound ‘ANA-1’ was shown to be effective in an influenza mouse model [59]. Cocrystallization or resistance studies with these PPI inhibitors are required to understand their precise binding mode within PA_C .

Nucleoprotein-binding agents

The viral nucleoprotein (NP) is an attractive drug target since it is well conserved and has multiple regulatory and structural functions. To cover the viral RNA, it forms NP oligomers in which the tail loop inserts into the body domain of another NP molecule, and the RNA is bound in a groove lined by basic residues. Diverse small-molecule NP inhibitors with activity in virus-infected cell cultures have been discovered (see [60] and references therein). For the NP-aggregating agent nucleozin, the binding site in NP was identified by cocrystallization (Figure 2) and viral resistance studies [61]. Nucleozin displayed nice efficacy in influenza mouse models, yet is inactive against some influenza strains with pre-existing resistance. This issue could be solved by targeting the highly

conserved RNA binding groove, such as seen with the known pharmacological agents curcumin [62[•]] and naproxen [63].

Conclusion

During the past five years, enormous progress has been made in revealing the structure of several conserved influenza protein domains, creating ample opportunities for inhibitor design. We here briefly reviewed some concepts targeting the viral hemagglutinin, polymerase complex or nucleoprotein, for which antiviral leads are in the (pre)clinical stage. It can be anticipated that new influenza blockers will achieve market approval within the forthcoming years. Given the high sequence variation and mutability of influenza A and B viruses, we need an arsenal of drugs with different action mechanisms and resistance profiles, to address severe influenza infections in a population that is ageing and highly susceptible to new influenza viruses with pandemic potential.

Conflict of interest statement

Nothing declared.

Acknowledgments

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- of special interest
- of outstanding interest

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